THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1986 by The American Society of Biological Chemists, Inc.

Vol. 261, No. 9, Issue of March 25, pp. 4229–4238, 1986
Printed in U.S.A.

Electroblotting onto Activated Glass

HIGH EFFICIENCY PREPARATION OF PROTEINS FROM ANALYTICAL SODIUM DODECYL SULFATE-POLYACRYLAMIDE GELS FOR DIRECT SEQUENCE ANALYSIS*

(Received for publication, July 22, 1985)

Ruedi H. Aebersold‡, David B. Teplow, Leroy E. Hood, and Stephen B. H. Kent§

From the Division of Biology 147-75, California Institute of Technology, Pasadena, California 91125

We have developed a new method for the isolation of proteins for microsequencing. It consists of electrophoretic transfer (electroblotting) of proteins or their cleavage fragments onto activated glass filter paper sheets immediately after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins are immobilized on the glass fiber sheets by ionic interactions or by covalent attachment. A wide range of proteins can be prepared in this fashion with no apparent restriction due to solubility, size, charge, or other intrinsic properties of the proteins. As little as 50 ng of the transferred proteins can be detected using Coomassie Blue or fluorescent dye staining procedures and even smaller amounts of radiolabeled proteins by autoradiography. After detection, the protein-containing bands or spots are cut out and inserted directly into a gas-phase sequenator. The piece of glass fiber sheet acts as a support for the protein during the sequencing. Amounts of protein in the 5- to 150-pmol range can be sequenced, and extended runs can be obtained from the blotted samples because of improved stepwise yields and lower backgrounds. The method has been successfully applied to the sequencing of a variety of proteins and peptides isolated from one-dimensional and two-dimensional polyacrylamide gels.

Protein sequence analysis is important in modern molecular biology. Partial amino acid sequence data are frequently used to assist in the isolation of the gene coding for a particular protein and to confirm that the correct gene has been isolated (1, 2). More extensive amino acid sequence data can be used to cross-check sequences determined at the nucleic acid level and to establish the structure of the mature translation product of the gene (3). Often, only very limited amounts of biologically interesting proteins are available (4). For this reason, there is great interest in highly sensitive methods for isolating and sequencing proteins.

Currently, as little as 10–20 pmol (0.5–1 μg for a 50,000-dalton molecule) of protein can be sequenced using the gas-phase sequenator (5). Isolation of such small amounts of pure protein in a form suitable for sequencing presents a considerable technical challenge. The most commonly used procedures are the separation of microgram amounts of proteins on SDS[1]-polyacrylamide slab gels, followed by Coomassie Blue staining and electroelution of the stained protein from the gel slice (6), or reverse phase HPLC using gradient elution with volatile solvents (7). These techniques are generally applicable, although they each can give variable recoveries of particular proteins. They can also be technically difficult, quite time consuming, or require expensive and specialized equipment.

It is our aim to use the most sensitive analytical techniques as preparative methods for protein sequence analysis. Such methodology will require the improvement of all aspects of microsequencing, but especially those involving the isolation of nanogram (subpicomole) amounts of protein. Suitable techniques will be simple, rapid, applicable to a wide range of proteins, have high resolution and sensitivity, and produce excellent overall recoveries and sequencing yields of the protein samples.

In this paper we describe methods for the direct electrophoretic transfer of proteins from SDS-polyacrylamide gels onto chemically activated glass fiber sheets and subsequent high sensitivity detection of the blotted proteins. Proteins isolated in this way are suitable for use in the gas-phase microsequenator without further manipulation. The general utility of the method is illustrated by sequence data from a wide variety of proteins and peptides prepared from one- and two-dimensional SDS gels.

EXPERIMENTAL PROCEDURES

Materials

The glass filter paper was Whatman GF/C or GF/F. This is available in large rectangular sheets but can be obtained more cheaply in large circles. Sequenal-grade trifluoroacetic acid was purchased from Pierce Chemical Co., or was prepared from technical-grade stock (Halocarbon Chemicals, Hackensack, NJ) by distillation over Alumina Woelm N, Type I (Woelm Pharma, FRG). 3-Aminopropyltriethoxysilane and 1,4-phenylenediisothiocyanate (DITC) were obtained from Pierce Chemical Co. *N*-trimethoxysilylpropyl-*N,N,N*-trimethylammonium chloride was from Petrach Systems Inc., Bristol, PA. Nonidet P-40 was obtained from Particle Data Laboratories, Elmhurst, IL. Analytical reagent-grade glacial acetic acid was from J. T. Baker Chemical Co. Chemicals for polyacrylamide gel electrophoresis were all electrophoresis-grade (6). Reagent-grade dithiothreitol was purchased from Aldrich. Standard blotting apparatus was used. In our case the Bio-Rad "Trans-Blot" cell and Model 250/2.5 constant voltage power supply. Coomassie Brilliant Blue R-250 was from

* This work was supported by research grants from the National Institutes of Health, the National Science Foundation (Biol. Inst. Grant DMB-8500298), the Monsanto Company, and Upjohn Pharmaceuticals. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a training fellowship from the Swiss National Foundation.

§ Made an oral presentation of the results contained in this paper at the Methods in Protein Sequence Analysis meeting in Cambridge, United Kingdom August 2, 1984.

[1] The abbreviations used are: SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; DITC, 1,4-phenylenediisothiocyanate; PITC, phenylisothiocyanate; PTH, phenylthiohydantoin; DPTU, diphenylthiourea; dabsyl, 4′-dimethylaminoazobenzene-1-sulfonyl chloride.

Serva. 3,3′-Dipentyloxacarbocyanine iodide was purchased from Molecular Probes, Inc., Junction City, OR. Proteins were ^{125}I-labeled by the chloramine-T method (8). Specific activities of radiolabeled proteins were determined by gamma scintillation counting, without correction, of trichloroacetic acid-precipitable material, and amino acid analysis of acid hydrolyzates was performed using the dabsyl chloride method (9).

Methods

SDS-Polyacrylamide Gel Electrophoresis—Minigels (9 cm × 9 cm × 0.5 mm) were run essentially according to Laemmli (10). Modified procedures and specific precautions to prevent amino-terminal blocking of proteins have been described (6), except that thioglycolate was not always used. Two-dimensional gels were run according to the method of O'Farrell (11).

Acid Blotting—(i) Activation of Glass Fiber Sheets

Acid Etching—GF/C or GF/F sheets or circles were immersed in neat trifluoroacetic acid in a covered Petri dish and kept for 1 h at room temperature, after which the trifluoroacetic acid was decanted. If more than one sheet was used at a time, extreme care was taken to remove any air bubbles between sheets so that the trifluoroacetic acid contacted the glass fiber sheets completely and uniformly. This was best achieved by sliding the sheets into the trifluoroacetic acid one at a time. The glass fiber sheets were dried by blowing a stream of cold air over them until no traces of trifluoroacetic acid remained. Alternatively, after preliminary removal of trifluoroacetic acid by a cold air stream, traces of trifluoroacetic acid were removed by placing them under vacuum over KOH. A KOH trap was mounted between the vacuum pump and the desiccator. The drying time varied from 1 to a few hours, depending on the vacuum, number of sheets, and how thorough the preliminary removal of trifluoroacetic acid was. It was essential that the dried sheets contained no traces of trifluoroacetic acid. Activated sheets remained usable for at least several weeks when stored at room temperature in a glass container (*e.g.* a covered Petri dish). Because of the corrosive nature of trifluoroacetic acid, all manipulations were performed in an efficient fume hood using gloves and eye protection.

(ii) Electroblotting from SDS-Polyacrylamide Gels

Immediately after the electrophoresis, SDS was displaced from the proteins by immersing the gels in 0.5% (v/v) acetic acid containing 0.5% (v/v) Nonidet P-40, for 10 min at room temperature. Before the sandwich for electroblotting was assembled (Fig. 1), each component was soaked in the blotting solution (1–5% (v/v) acetic acid in water containing 0.5% Nonidet P-40). The sandwich consisted of the following components, oriented from the negative to the positive pole of the blotting buffer chamber (Fig. 1): sponge; Whatman No. 3MM paper, cut the same size as the sponge; trifluoroacetic acid-activated GF/C or GF/F (backup sheet); trifluoroacetic acid-activated GF/C or GF/F sheet; the gel; Whatman No. 3MM paper, cut the same size as the sponge; sponge. Care was taken to eliminate any bubbles between the gel and the activated glass fiber sheet. This was best achieved by pressing with a glass rod or spatula on the glass fiber sheet placed on top of the gel. The sandwich was then assembled and placed into the blotting chamber, which was filled with solution equilibrated to 0–5 °C. The blotting solution was usually 1% acetic acid containing 0.5% Nonidet P-40. However, for proteins that were difficult to transfer, up to 5% acetic acid (with 0.5% Nonidet P-40) was used. To minimize acid-catalyzed cleavage of the proteins (12), acid concentrations were kept as low as possible. Blotting was performed at 70 V, corresponding to 250–600 mA (depending on the dimensions of the blotting chamber and concentration of the buffer) for 3–12 h at 0–5 °C. To maintain this temperature, the outside of the blotting chamber was cooled with ice water, or a cooling coil was immersed in the blotting solution. The solution in the blotting chamber was magnetically stirred throughout the procedure.

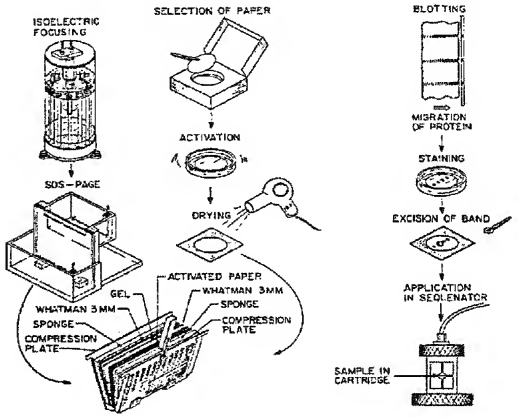

FIG. 1. **Principles of blotting.** Proteins to be electroblotted may be prepared by one- and/or two-dimensional gel electrophoresis. After electrophoresis, the slab gel is mounted in a standard blotting apparatus against a sheet of chemically activated glass fiber filter paper. The proteins are electrophoretically transferred onto the paper and then visualized by staining with Coomassie Blue, fluorescent dyes, or by autoradiography. The stained protein bands are excised and loaded directly into a gas-phase sequenator.

(iii) Detection of Proteins

Proteins that had been electroblotted onto the trifluoroacetic acid-activated glass fiber sheets were detected by staining with Coomassie Blue. The glass fiber sheet was placed in staining solution [0.5% (w/v) Coomassie Blue R-250, 30% (v/v) isopropyl alcohol, 10% (v/v) acetic acid, in distilled water] for 2 min. Destaining was performed under warm (40 °C) running distilled water, or by shaking the blot in distilled water or destaining solution (16.5% (v/v) methanol, 5% (v/v) acetic acid, in distilled water). Thorough destaining was necessary to avoid artifact peaks in phenylthiohydantoin (PTH) analysis after sequencing. Protein bands treated with destaining solution were washed with distilled water to remove all traces of acid before storage.

(iv) Storage

Electroblotted protein bands were stored at −20 °C, or dry in a desiccator at room temperature. Blotted samples could be stored for at least 2 months prior to sequencing.

High pH Blotting—(i) Activation of Glass Fiber Sheets

Aminopropyl and Quaternary Ammonium Glass Fiber Sheets—Trifluoroacetic acid-etched GF/C or GF/F glass fiber sheets were immersed in freshly made 2% (v/v) solutions of 3-aminopropyltriethoxysilane or *N*-trimethoxysilylpropyl-*N,N,N*-trimethylammonium chloride in 95% acetone in water for 2 min in a glass Petri dish. The sheets were then washed at least 10 times in acetone (5 min each wash) on a shaking platform to remove excess silanizing reagent. Curing of the silane linkage (13) was achieved by drying the sheets supported on Whatman No. 1 paper in an oven for 45 min at 110 °C. Deposition of 3-aminopropyl groups on the glass fiber sheets and the completeness of the washing process were monitored by quantitative ninhydrin determination of free amino groups on the sheets and in the washes. The acetone washes were dried down in 10 × 75-mm glass tubes under a stream of N_2, or a 5-mm diameter disc of the cured aminopropyl-glass fiber paper was placed in a glass tube of the same dimensions. The quantitative ninhydrin determination was carried out as previously described (14). Typical quantities of bound NH_2-groups were between 8–12 nmol/mg of glass. Washing was continued until no NH_2 groups could be detected in 3 ml of the washing supernatant.

DITC-glass Fiber Paper—DITC-glass fiber sheets were prepared by modifying the procedure described by Machleidt and Wachter (15) for reacting aminopropyl glass beads. Dried aminopropyl-glass fiber sheets (GF/C or GF/F) prepared as described above were incubated for 2 h at 22 °C in a Petri dish on a shaker with a 10-fold molar excess of DITC over amino groups (determined by the quantitative ninhydrin reaction). DITC was dissolved in 10 ml of 10% (v/v) pyridine in *N,N*-dimethyl formamide for a 15-cm diameter sheet. Excess reagent was removed by three cycles of successive washes of the glass fiber sheet with methanol and acetone. Completeness of the reaction was estimated by determination of residual amino groups using the quantitative ninhydrin reaction.

(ii) Electroblotting from SDS-Polyacrylamide Gels

Immediately after electrophoresis, the sandwich was assembled and electroblotting performed as described for acid blotting, with the

following exceptions: 1) quaternary ammonium-glass fiber paper (QA-glass), aminopropyl-glass fiber paper (AP-glass) or DITC-glass were used instead of trifluoroacetic acid-activated glass fiber paper; 2) the sandwich was oriented such that the activated paper was on the positive (anode) side of the gel; 3) the blotting buffer was 25 mM Tris-HCl, pH 8.3, containing 10 mM glycine and 0.5 mM dithiothreitol (not suitable for DITC-glass paper) or 25 mM Tris-HCl, pH 8.3, containing 0.5 mM dithiothreitol; 4) blotting was performed for 2 h at 50 V (400–700 mA) at ~2–5 °C for 12.5% total acrylamide, 0.5-mm thick gels. For thicker gels, or gels with a higher per cent total acrylamide, blotting time was increased.

(iii) Detection

Proteins electroblotted onto chemically derivatized glass paper at high pH were detected by a novel fluorescent staining method, using 3,3′-dipentyloxacarbocyanine iodide, according to the following procedure. The blotted paper was partially dried under a stream of air or at 40 °C in an oven for approximately 20 min or by repeatedly pressing against fresh sheets of Whatman No. 1 paper. The staining solution consisted of 3 mg of 3,3′-dipentyloxacarbocyanine iodide completely dissolved in 3 ml of methanol by vigorous mixing, which was then added to 27 ml of 0.05 M $NaHCO_3$, pH 8.2–8.6. The partially dried blot was added to the staining solution in a Petri dish and shaken for 15 min at room temperature. Each blot was stained separately in fresh staining solution. Protein quantities of more than 1 μg could be immediately located as orange bands against a background of the yellow dye solution. Smaller amounts of proteins (50 ng–1 μg) were detected on the wet blot, using 254 nm UV light, as fluorescent green-yellow bands on a dark background. Destaining was generally unnecessary but could be performed by washing with the same buffer without the added dye. Alternatively, when samples were radioiodinated, the blot was autoradiographed at −70 °C or room temperature using Kodak XAR-5 x-ray film.

(iv) Storage

Excised protein bands were stored at −20 °C in 1.5-ml microcentrifuge tubes for more than 2 months prior to sequencing without noticeable changes in sequencing efficiency. As an additional precaution, protein bands to be stored for a prolonged period of time were completely destained by soaking 2–3 times for 5 min each in 1 ml of sequencing grade ethyl acetate to remove the dye.

Sequencing of Immobilized Proteins

Protein bands, detected after Coomassie or fluorescent staining procedures, or by autoradiography, were cut out of the glass fiber sheets and placed in the cartridge of a gas-phase sequenator without any further treatment. Well resolved single bands were cut out as discs to fit directly into the upper block of the cartridge. Narrowly spaced bands on the blot were cut out, and several pieces (*e.g.* from different lanes in a gel) were loaded on top of the Zitex support in the cartridge. If small pieces or strips of glass fiber paper were placed in the cartridge, they were covered by a disc of untreated GF/C to avoid channeling of reagents and solvents during sequencing. The insertion of more than two complete layers of glass fiber strips into the cartridge impeded the flow of reagents and gave incomplete reactions and poor sequencing results. Thus, particular attention was paid to reagent delivery rates, especially S2 (ethyl acetate), for the first few deliveries in a sequencing run.

The protein sequence determination was performed as described (5), with HCl-methanol conversion. The sequencing program was started by a subroutine consisting of a 10-min trifluoroacetic acid treatment followed by a 90-second ethyl acetate or butyl chloride wash before the first coupling was performed. These washes completely removed the 3,3′-dipentyloxacarbocyanine iodide but not Coomassie Blue. The resulting PTH derivatives were analyzed by HPLC on an IBM cyano column essentially as described by Hunkapiller *et al.* (16) except that 5–7% (v/v) tetrahydrofuran was added to the "A" buffer and the pH was adjusted to 5.1. PTHs were quantitated by comparison of peak heights with standards.

Proteins and peptides were also sequenced, without prior electroblotting, by direct application of the dissolved sample onto discs of chemically activated glass fiber paper. Both the use of Polybrene and precycling of the activated disc were unnecessary.

RESULTS AND DISCUSSION

Principle of the Method—The fundamentals of electroblotting are shown in Fig. 1. The method consists of four steps: separation of proteins by SDS-polyacrylamide gel electrophoresis; their electrophoretic transfer from the unstained gel to a sheet of suitably activated glass fiber "paper"; detection of the transferred proteins; and the excision of the protein-containing bands or spots on the glass fiber support and their direct insertion into the sequenator reaction cartridge.

Selection and Activation of the Support—In the past, a variety of supports have been used for analytical electroblotting. These include nitrocellulose (17), diazophenyl papers (18), and charge-modified nylon sheets (19). We investigated the suitability of these supports for use in the gas-phase protein sequenator. During the sequencing chemistry the nitrocellulose dissolved, while the charge-modified nylon collapsed into a solid pellet. In preliminary experiments, diazophenyl paper appeared to be compatible with use in the gas-phase sequenator. However, we decided to focus on the development of glass fiber paper as a support for electroblotting because glass fiber discs are now routinely used as the support for proteins or peptides in the gas-phase sequenator. The glass fiber discs are completely stable to the conditions of sequencing and are not known to interfere with the chemistry or introduce background contaminants in the subsequent PTH analyses.

Glass fiber paper is available in circular and rectangular sheets for use as a filter medium. A variety of weights, thicknesses, and densities is available. We found that Whatman GF/C and GF/F were suitable for our purposes. GF/F has a higher capacity but is also somewhat more bulky.

The untreated glass fiber sheets have a very limited ability to adsorb proteins. Based on anecdotal evidence and very limited reports (20, 21), we investigated acid etching as a means to increase the capacity of the glass fiber. We found that after treatment with trifluoroacetic acid the glass fiber sheets had considerable capacity for adsorbing proteins (7–10 $\mu g/cm^2$). The mechanism of binding appeared to be noncovalent as the proteins were easily eluted with dilute solutions of SDS or with methanol. Furthermore, excess SDS interfered with the trapping of the proteins during the blotting. From this we deduced that the mechanism of adsorption of the proteins was ionic interaction of the net positively charged protein with negative charges on the glass sheet (Fig. 2). These negatively charged sites were presumably revealed by the acid etching of the glass surface.

Having established that SDS interfered with the binding of proteins to acid-etched glass, we set out to develop a glass fiber support that would take advantage of the presence of SDS on the proteins to trap them in a form suitable for sequencing: *i.e.* a positively charged support. To this end we covalently modified acid-etched glass sheets with amine-containing silanes (Fig. 3) (15, 22). The acid etching of the glass fiber sheet increased the extent of the chemical modification and the capacity of the resulting supports for adsorbing proteins. Without acid etching, aminopropyltriethoxysilane-modified GF/F gave an average of 5 μmol amino groups/g (as determined by the quantitative ninhydrin reaction). With prior acid etching, the same reaction gave an average of 10 μmol/g. The resulting aminopropyl-glass fiber sheets could be used in electroblotting SDS-coated proteins at high pH (Fig. 2). The aminopropyl groups ($pK_a = 10.5$) retain their positive charge at pH 8 in the blotting procedure, and the silane linkage is stable to sequencing chemistry (15). They do not interfere with sequencing using standard protocols (5) although they were converted to uncharged phenylthiocarbamyl

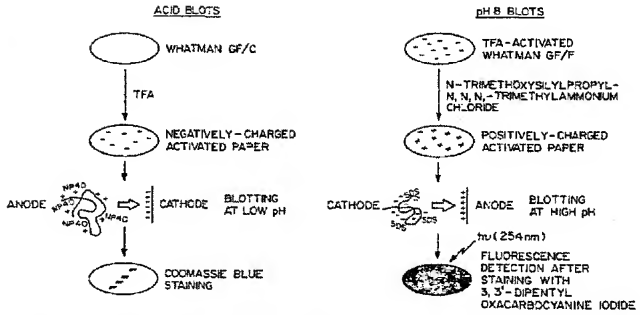

FIG. 2. **Mechanisms of protein transfer and detection.** Acid blotting is performed using Whatman glass fiber filter paper discs activated by immersion in anhydrous trifluoroacetic acid. After complete removal of excess acid, the activated paper is placed in the blotting apparatus next to a polyacrylamide gel and electrophoresis performed in dilute acetic acid containing Nonidet P-40. At this low pH, the proteins have a net positive charge and migrate toward the cathode onto the paper. The transferred proteins are visualized by staining with Coomassie Blue. Blotting at high pH (8.3) is performed similarly, except that the trifluoroacetic acid-activated paper is further derivatized with a quaternary ammonium compound and the proteins, due to the presence of SDS, migrate toward the anode onto the paper. The proteins are visualized by treatment with a fluorescent dye and irradiation with a short wave (254 mm) UV lamp. TFA, trifluoroacetic acid.

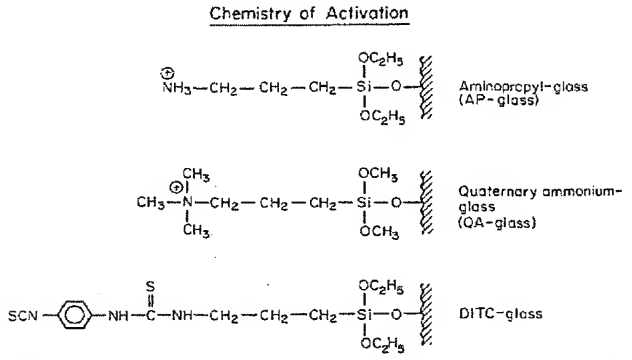

FIG. 3. **Chemistry of activation.** 3-Aminopropyltriethoxysilane is used to produce glass fiber supports containing aminopropyl groups (AP-glass) capable of ionic interactions with proteins and as substrates for further derivatizations. *N*-trimethoxysilylpropyl-*N,N,N*-trimethylammonium chloride is used to produce derivatized glass fiber supports containing quaternary ammonium functions (QA-glass) which can participate in ionic interactions with proteins. 1,4-Phenylenediisothiocyanate reacts with AP-glass to produce glass fiber supports to which proteins and peptides may be covalently linked. All support chemistries are compatible with gas-phase sequencing technologies.

derivatives in the first PITC coupling step, as shown by the quantitative ninhydrin reaction. Despite this loss of the charged amino group, there was no apparent increase in extractive losses of the protein during sequencing. Because of this observation, the mechanism of retention of the protein on the glass fiber support during sequencing must be different from the mechanism of initial trapping during electroblotting and probably involves ionic interactions with both positive and negative charges revealed on the glass by the repeated acid etching during trifluoroacetic acid exposure in the sequencing program.

We also developed a glass fiber support that would remain positively charged throughout the sequencing operations. Reaction of the glass fiber sheets with *N*-trimethoxysilylpropyl-*N,N,N*-trimethylammonium chloride resulted in a quaternary ammonium glass fiber sheet (Fig. 3). Again, prior acid etching was used to increase the capacity of the QA-glass sheets for adsorbed protein. The quaternary ammonium function cannot react with PITC and, therefore, remains positively charged at all times. This derivative performed extremely well in electroblotting at high pH and in sequencing and for these reasons became our support of choice. The capacity of QA-glass for protein is comparable to that of acid-etched glass (7–10 $\mu g/cm^2$).

The aminopropyl-glass fiber sheets discussed above are an ideal starting point for the introduction of a wide variety of chemical functional groups. As an example of this, we have prepared DITC-glass fiber sheets by reaction of the AP-glass with DITC (Fig. 3). The resulting DITC-glass has allowed us to use electroblotting to covalently immobilize proteins for sequencing (15).

Electroblotting—We have used blotting in dilute acetic acid onto acid-etched glass to prepare a wide variety of proteins for sequencing. Fig. 4 shows an experiment in which five proteins, with molecular weights ranging from 14,000 to 68,000, were electroblotted under these conditions. The intense staining of the blot and the lack of staining of the gel demonstrate that the transfer of the proteins has occurred with high efficiency. Further work showed that it was critical that all SDS be removed from the proteins prior to the electroblotting. This was done by soaking the gel in a neutral detergent such as Nonidet P-40, before blotting, to displace the SDS but maintain the protein in solution. If SDS removal is incomplete, the protein will tend to remain in the gel or move to the anode rather than to the cathode, resulting in very low transfer efficiencies. Furthermore, as discussed above, the SDS can interfere with the ionic trapping of the protein in the acid-etched glass.

Proteins blotted onto the acid-etched glass fiber sheets can be detected by Coomassie Blue staining. As can be seen in Fig. 4 by comparing the intensity of the stained spots on the reference gel with those on the blot, there is enhanced sensitivity of detection for blotted proteins. The detection limit seems to be about 50 ng, about 5–10 times more sensitive than Coomassie Blue staining of proteins in gels. As has been previously observed (23), the staining and destaining are also much more rapid for the blotted proteins. Note that because the proteins are blotted before staining, the staining procedure cannot interfere with transfer of the protein. Furthermore, provided all excess Coomassie Blue is washed from the surrounding glass fiber sheet, the stained protein sequences without interference from the dye, which remains bound to the protein throughout the sequencing process.

The efficiency of transfer of proteins from SDS-polyacrylamide gels to the acid-etched glass fiber sheets was investigated using radiolabeled proteins. The results are shown in Table I. Recoveries varied from 9 to 96% and showed little correlation with the pI of the proteins. Similar variation in recoveries was observed with a large number of other proteins. There is a slight correlation with molecular size: in general, the larger proteins tend to transfer very poorly, although there are notable exceptions to this rule of thumb, as shown in the Table. This variable and often low recovery of proteins limits the general usefulness of the acid blotting procedure, even though it has the virtue of extreme simplicity. Blotting onto acid-etched glass sheets at low pH from non-SDS containing gels does not show this variation in recoveries of blotted proteins, and this procedure has proven to be generally applicable to the high yield preparation of proteins for se-

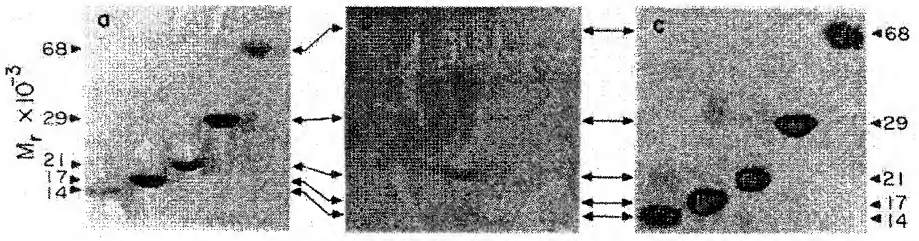

FIG. 4. **Electroblotting at low pH.** Bovine serum albumin (68 kDa), bovine carbonic anhydrase (29 kDa), soybean trypsin inhibitor (21 kDa), sperm whale myoglobin (17 kDa), and bovine α-lactalbumin (14 kDa) were each loaded onto two gels and then subjected to SDS-PAGE (12.5% T). One gel was then blotted at low pH onto trifluoroacetic acid-activated GF/C paper, then this gel (blotted gel) (*b*), the paper (blot) (*c*) and the second gel (reference gel) (*a*) were all stained with Coomassie Blue.

TABLE I

Blotting efficiency from SDS-polyacrylamide gels

Protein			Low pH[a]		High pH[b]	
			Found on blot	Found in gel	Found on blot	Found in gel
	$M_r \times 10^{-3}$	*pI*		%		
Bovine α-lactalbumin	14	4.6	68	<1	94	<5
Sperm whale myoglobin	17	8.3	78	<1	75	<5
Soybean trypsin inhibitor	21	4.5	42	25	99	<5
Bovine carbonic anhydrase	29	5.8	96	5	74	<5
Bovine serum albumin	68	4.9	77	<1	76	<5
Rabbit phosphorylase *b*	97	5.5–6.3	33	66	ND[c]	ND
E. coli β-galactosidase	116	4.5	9	69	81	<5

[a] 5 μg of each protein loaded onto the gel.
[b] 3 μg of each protein loaded onto the gel.
[c] ND, not determined.

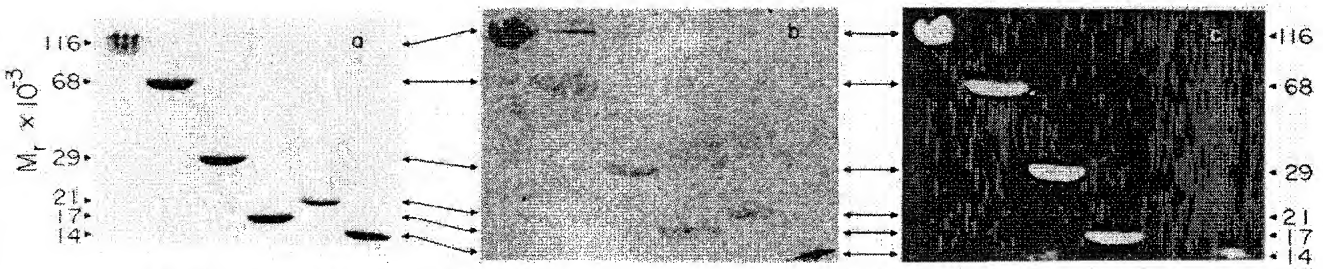

FIG. 5. **Electroblotting at high pH.** *Escherichia coli* β-galactosidase (116 kDa) was added to the set of proteins discussed in Fig. 4, and then each of the samples was loaded onto two gels and subjected to SDS-PAGE (12.5% total acrylamide). One gel was blotted at pH 8.3 onto quaternary ammonium activated GF/F paper (QA-glass), and then this gel (blotted gel) (*b*) and the second gel (reference) (*a*) were stained with Coomassie Blue. The paper (blot) (*c*) was stained with 3,3′-dipentyloxacarbocyanine iodide and photographed under 254 nm UV light.

quencing from "Immobiline" isoelectric focusing gels.[2]

We believe that the erratic recoveries from SDS-gels are due to retention of SDS to different extents by different proteins and to differences in solubility after SDS replacement. It may be that other neutral detergents could be used to more completely and generally displace the SDS from the proteins before blotting, while still maintaining solubility. Rather than explore this route, we chose to use to our advantage the fact that in SDS-PAGE proteins are coated with SDS.

When positively charged glass fiber sheets were used for electroblotting at pH 8.3, much more reproducible results were obtained (Fig. 5). This experiment included the same five proteins shown in Fig. 4, plus β-galactosidase, 116 kDa. No pretreatment of the gel before blotting was required. Buffer salts or other chemical species present after SDS-PAGE did not interfere with either the blotting or with subsequent sequencing. The blotting buffer used at pH 8.3 was either Tris-glycine or Tris-HCl, both containing dithiothreitol. Transfer of the proteins was quite rapid, the standard time typically being 2 h for 0.5-mm thick gels.

Proteins blotted onto positively charged glass fiber sheets could not be detected using Coomassie Blue because the whole glass fiber sheet stained dark blue. It was therefore necessary to develop a new staining method that would be compatible with the presence of the positively charged functionalities in the sheets as well as with subsequent sequencing of the proteins. This method used the fluorescent dye 3,3′-dipentyloxacarbocyanine iodide, which shows a change in its absorption and emission spectra upon binding to proteins. This dye has previously been used for fluorescence studies of membranes (24). Detection was based on hydrophobic interaction

[2] R. H. Aebersold, D. B. Teplow, L. E. Hood, and S. B. H. Kent, manuscript in preparation.

between the proteins and the two pentyl chains attached to the dye. Various length alkyl chains were investigated, and the five-carbon chain offered an optimum combination of sensitivity and selectivity for proteins. The staining procedure is a single-step one in which the partially dry blot is soaked in the light yellow dye solution for 15 min at room temperature. Proteins present in amounts of more than 1 μg are immediately visible as orange spots due to a change in the absorption spectrum of the dye. Under 254 nm UV light, proteins show up as strongly fluorescent spots. Detection limits vary slightly from protein to protein but typically range from 20–50 ng. Destaining had little effect on detection sensitivity. It was not necessary for subsequent sequencing since the dye was quantitatively removed by the first ethyl acetate or butyl chloride wash.

The efficiency of blotting of proteins from SDS-polyacrylamide gels at high pH is shown in Table I. Recoveries of the protein standards were uniformly high, ranging from 74 to 99%. Our experience with a wide range of proteins (Table III) and with complex mixtures containing many different protein species has confirmed this observation. Yields between 70 and 100% are routinely obtained, even large (100 to 200-kDa) proteins transferring with high efficiency. We have observed comparable recoveries from gels of low and high per cent total acrylamide, and from gradient gels. Thus, the high pH blotting procedure is a general method for the preparation of proteins for sequencing following SDS-PAGE.

Sequencing—Protein samples prepared by both blotting methods sequenced efficiently. In either method, the protein-containing regions of the stained glass fiber sheets were cut out and placed directly in the cartridge of the gas-phase sequenator. Using radiolabeled proteins of known specific activity (determined by amino acid analysis) initial yields of sequencable material were determined for several proteins (Table II). Both methods gave an initial PTH signal corresponding to 61–76% of the protein present. The repetitive yields were measured for the same set of proteins based on the amount of PTH present in successive cycles of the Edman degradation. As can be seen in Table II, repetitive stepwise yields were typically 93–94% for amounts of protein in the 50-pmol range. This is about 2% per cycle better than we routinely obtain for the same proteins on Polybrene-GF/C discs. Such an improvement in repetitive yields has dramatic effects on our ability to carry out extended sequence determinations on limiting amounts of material. It would, for example, allow the determination of 33 residues of sequence where previously only 25 residues could be determined (for 20 pmol of protein with a detection limit of 2 pmol).

The use of radiolabeled proteins enabled us to quantitate protein losses from the glass fiber support during sequencing. There were slightly smaller losses from the acid-etched support than from the covalently-modified glass fiber support, but both were acceptably low at about 1% per cycle (Table II). The remaining 5% per cycle observed reduction in sequencable yield is likely to be due to chain terminating side reactions in the Edman chemistry (25).

In addition to the improved repetitive stepwise yields, sequencing of blotted proteins prepared by either methodology gave PTH analyses with extraordinarily low backgrounds of by-product peaks. This is dramatically illustrated in Fig. 6 where the PTH analyses from the first cycles of four different sequencing runs are shown. Even where high pH blotting in 25 mM Tris-HCl, 192 mM glycine, 0.5 mM dithiothreitol (which is not routinely used) onto QA-glass fiber paper has been carried out, there is very little contamination of the PTH derivatives with glycine or with phenylthiocarbamyl dimethylamine (PTC-DMA), diphenylthiourea (DPTU), or diphenylurea. Levels of the first two are typically the equivalent of a PTH signal of 2 pmol or less, while the level of DPTU plus diphenylurea is usually the equivalent of 10–20 pmol. Buffers for PTH separation are tuned so that peaks of contaminants are baseline-resolved from the known elution positions of PTHs (16). Apparently, much of the background normally seen in HPLC analysis of PTH samples from the gas-phase sequenator must arise because of the use of Polybrene to immobilize the sample.

This combination of high initial yields, high repetitive stepwise yields, and very low backgrounds in the PTH analysis has allowed us to undertake sequencing of very small amounts of proteins. The first two traces of a sequencing run on myoglobin electroblotted at high pH (3 pmol sequencable material) are shown in Fig. 7. Even small PTH signals are readily detected because of the absence of extraneous peaks. Even determination of NH_2-terminal sequences of large proteins can be carried out at very low levels (Table III).

Where the protein has been prepared by means other than SDS-PAGE it can be directly applied to trifluoroacetic acid-etched, AP-, or QA-glass discs in much the same way that samples are applied to Polybrene-coated discs. We have carried out sequence determinations on a variety of proteins and peptides in this way. For example, it was possible to determine the sequence of 8 of the 9 residues of 60 pmol of [Arg^8] vasopressin spotted onto trifluoroacetic acid-etched GF/C in dilute acetic acid. Peptides separated by reverse phase HPLC can be directly spotted in the solvents in which they are recovered. Our experience with the direct application of peptides is still relatively limited and many more examples will have to be investigated before the generality of direct application of small peptides for sequencing can be established. These three supports show about the same tolerance as Polybrene-coated discs to solvents used to apply the sample. Even protein solutions containing SDS or buffer salts can be used for direct application of the sample onto QA-glass for sequencing, although very high concentrations of either may interfere with retention of the protein.

Applications—We have applied the techniques described above to the determination of NH_2-terminal amino acid sequences of a large number of protein and peptide samples.

TABLE II
Sequencing yields from blotted proteins

Protein	Low pH				High pH			
	Amount loaded	Initial yield	Repetitive yield	Sample loss/cycle	Amount loaded	Initial yield	Repetitive yield	Sample loss/cycle
	pmol		%		*pmol*		%	
Bovine α-lactalbumin	48	61	93	1.4	45	61	94	1.4
Sperm whale myoglobin	140	67	96	0.8	41	76	93	1.9
Soybean trypsin inhibitor	24	71	92	0.5	33	62	94	0.8
Bovine serum albumin	38	74	94	0	29	66	93	0.9

TABLE III

Proteins sequenced using the electroblotting technique

Protein		Sample preparation	Sequencable amount	Number of residues determined
	$M_r \times 10^{-3}$		*pmol*	
Rat liver gap junction chymotryptic fragment	10	low pH blot	7	9/14
Rat liver gap junction chymotryptic fragment	14	low pH blot	15	10/15
IgG H-chain	58	low pH blot	8	14/20
Mouse liver gap junction	28	high pH blot	12	16/18
Mouse liver membrane protein (gap junction associated)	21	high pH blot	5	10/16
Mouse liver membrane protein (gap junction associated)	16	high pH blot	140	19[a]
IgG heavy chain	58	high pH blot	110	20[a]
IgG light chain	28	high pH blot	12	13/17
Cholinergic differentiation factor	48	high pH blot	10	10/13
HF-treated cholinergic differentiation factor[e]	28	high pH blot	12	9/12
Soluble binding protein	150	high pH blot	33	21/24
Bovine serum albumin	68	high pH blot[b]	12	13/17
Soybean trypsin inhibitor	21	high pH blot[b]	11	12/13
Sperm whale myoglobin	17	high pH blot[b]	12	10/11
Bovine α-lactalbumin	14	high pH blot[b]	12	11/13
Yellow fever virus protein	45	spotted[c]	400	20[a]
μ-phage transposase	70	spotted[d]	5	8/11
Scrapie-associated protein cyanogen bromide fragment	1.8	spotted[c]	50	12/14
[Arg^8]Vasopressin	1	spotted[c]	60	8/9
Synthetic peptide	4.5	spotted[c]	400	32/40
DNA binding protein tryptic peptide		spotted[c]	25	12/13

[a] Run terminated at this point.
[b] From two-dimensional gel.
[c] Spotted onto QA-glass disc in water, dilute acid, or 0.1% trifluoroacetic acid-aqueous acetonitrile.
[d] Spotted onto QA-glass disc in 300 mM KCl.
[e] Chemically deglycosylated on a microscale with hydrogen fluoride (HF).

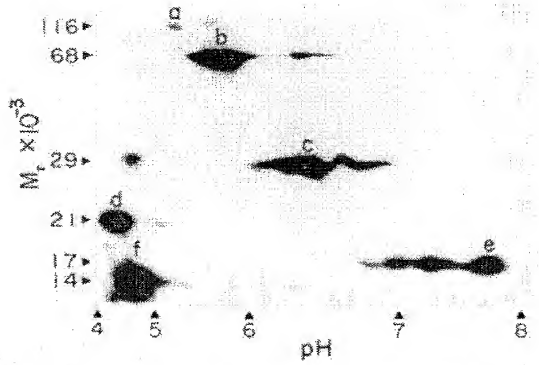

FIG. 8. **Electroblotting after two-dimensional SDS-PAGE.** A mixture containing the following radioiodinated proteins was subjected to two-dimensional SDS-PAGE, blotted at high pH onto QA-glass and autoradiographed. The *spots* on the pictured autoradiograph correspond to: (*a*) *E. coli* β-galactosidase, (*b*) bovine serum albumin, (*c*) bovine carbonic anhydrase, (*d*) soybean trypsin inhibitor, (*e*) sperm whale myoglobin, and (*f*) bovine α-lactalbumin. Using the autoradiograph as a template, *spots* were excised from the blot and loaded directly in a gas-phase sequenator.

currently limited by our inability to detect PTH derivatives below the 0.5–1-pmol range, thus limiting determination of unknown sequences to amounts of at least 3–5 pmol of sequencable protein. This is at the high end of the normal range for amounts of proteins separated on two-dimensional gels (26). When high sensitivity detection methods for PTH analysis (27) enable us to sequence at the 10- to 100-fmol level, two-dimensional gel electrophoresis and high pH blotting onto modified glass fiber sheets will be the most powerful general method for preparing proteins for NH_2-terminal sequence analysis.

Another application where the high pH electroblotting technique is useful involves "multi-dimensional" analysis to obtain internal amino acid sequence information from a protein. A mixture of proteins is separated by SDS-PAGE, and a gel slice containing the protein of interest is cut out. The protein is digested in the gel slice either chemically (28, 29) or enzymatically (30) and the resulting fragments applied to a second SDS-PAGE gel of higher per cent total acrylamide. After separation, all the fragments are blotted onto an activated glass fiber sheet and individually subjected to sequence analysis. This is a convenient gel analog of the multi-dimensional HPLC approach to obtaining internal sequence information from proteins (7).

Covalent attachment of a protein to a solid support offers greater flexibility in the choice of conditions for the Edman reaction (31), an important consideration in developing improved sequencing methods. In the past, use of solid-phase sequencing has been severely restricted by the lack of general methods of covalently immobilizing very small amounts of proteins in high yields. Blotting onto suitably activated glass fiber sheets offers a general method for the high yield covalent immobilization of proteins for solid-phase sequencing. Proteins electroblotted onto DITC-glass sheets at high pH become covalently attached through their α-amino groups and lysine ϵ-amino groups. We have found that such proteins sequence normally on the gas-phase sequenator, except for low yields of the NH_2-terminal amino acid and lysine residues.

Conclusions—We have described a novel approach to the

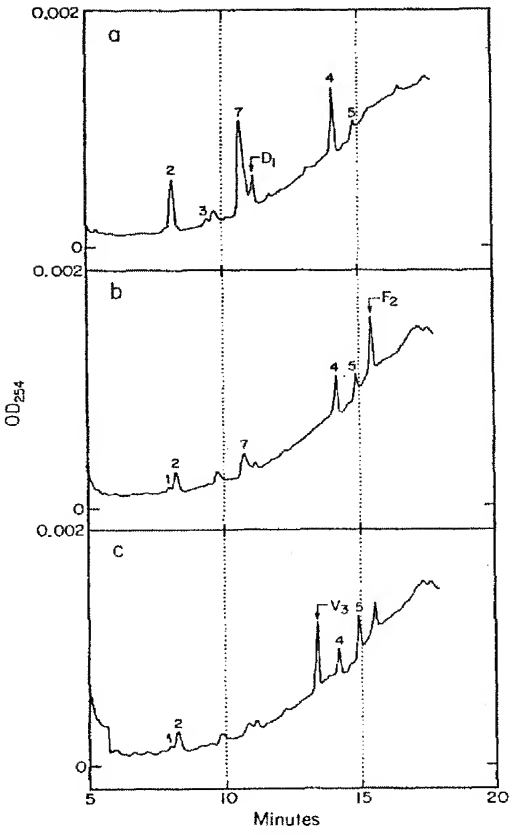

FIG. 9. **Sequence analysis of soybean trypsin inhibitor electroblotted after two-dimensional SDS-PAGE.** The portion of the blot discussed in Fig. 8 corresponding to *spot d* of the autoradiograph and containing soybean trypsin inhibitor was excised and loaded in a gas-phase sequenator. HPLC analyses of the PTH residues derived from amino acids 1–3 are presented in *panels a–c*, respectively. The *dotted vertical lines* are included for orientation when comparing the traces to each other. Labeled peaks as in Fig. 6: 7, second unidentified peak.

preparation, for the gas-phase sequenator, of proteins separated by SDS-PAGE. Electrophoretic transfer from polyacrylamide gels at pH 8.3 onto chemically activated glass fiber sheets, subsequent detection of the protein, and direct use of the glass fiber-bound protein in sequence determination offers a number of advantages over previous methods of isolating proteins for sequencing. The commonly used separation technique, SDS-PAGE, is highly resolving and generally applicable to a wide range of proteins. The electroblotting method is straightforward and does not require expensive or complex equipment. The protein is prepared for sequencing without ever being directly handled or removed from solution until it is attached to the support on which it is sequenced. In this form, it can be isolated without the extraordinary precautions often required to minimize sample losses. This gives high recoveries of even nanogram amounts of most proteins. Because the stained protein is sequenced *in situ* on the glass fiber blot, there is no doubt as to the presence or identity of the protein being sequenced. Protein samples prepared in this way are inserted directly into the sequenator without further manipulation.

We have also found the glass fiber supports to be useful for the direct application of proteins or peptides isolated by means other than SDS-PAGE. Analyses of the PTH derivatives resulting from sequencing samples blotted or spotted onto these glass fiber supports show unusually low amounts of PTC-DMA, DPTU, diphenylurea and other UV-absorbing contaminants, allowing higher sensitivity detection. This, combined with the improved stepwise yields, means that much longer sequences can be obtained from very small amounts of proteins.

Perhaps the greatest single advantage of the electroblotting procedure is that it involves the concurrent preparation of large numbers of proteins for sequencing. Depending on the samples, it is in principle possible to isolate hundreds of proteins for sequencing in a single operation from one polyacrylamide gel. This procedure also is applicable, without modification, to the isolation of proteins from two-dimensional gels, where simultaneous preparation of many more samples is now feasible.

Our goal has been to develop a general method for the high yield isolation of proteins in a covalently immobilized form suitable for use in protein sequencing. The methods described above represent an intermediate step toward this goal and should be of extraordinary utility in protein sequencing.

Acknowledgments—The contributions of Dr. Lloyd Smith, for suggesting the use of fluorescent dyes, and the expert technical contributions of John Kim, Eva Lujan, Wade Hines, and Chin Sook Kim are gratefully acknowledged.

REFERENCES

1. Prusiner, S. B., Groth, D. F., Bolton, D. C., Kent, S. B., and Hood, L. (1984) *Cell* **38**, 127–134
2. Oesch, B., Westaway, D., Wälchli, M., McKinley, M. P., Kent, S. B. H., Aebersold, R., Barry, R. A., Tempst, P., Teplow, D. B., Hood, L. E., Prusiner, S. B., and Weissmann, C. (1985) *Cell* **40**, 735–746
3. Clark-Lewis, I., Kent, S. B. H., and Schrader, J. W. (1984) *J. Biol. Chem.* **259**, 7488–7494
4. Ullrich, A., Bell, J. R., Chen, E. Y., Herrea, R., Petruzzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfield, C., Rosen, O. M., and Ramachandran, J. (1985) *Nature* **313**, 756–762
5. Hewick, R. M., Hunkapiller, M. W., Hood, L. E., and Dreyer, W. J. (1981) *J. Biol. Chem.* **256**, 7990–7997
6. Hunkapiller, M. W., Lujan, E., Ostrander, F., and Hood, L. E. (1983) *Methods Enzymol.* **91**, 227–236
7. Hunkapiller, M. W., Strickler, J. E., and Wilson, K. J. (1984) *Science* **226**, 304–311
8. Hunter, W. M., and Greenwood, T. C. (1962) *Nature* **194**, 495–496
9. Chang, J. Y., Knecht, R., and Braun, D. G. (1981) *Biochem. J.* **199**, 547–555
10. Laemmli, U. K. (1970) *Nature* **227**, 680–685
11. O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021
12. Poulson, K., Fraser, K. J., and Haber, E. (1972) *Proc. Natl. Acad. Sci. U. S. A.* **69**, 2495–2499
13. Pleuddemann, E. P. (1970) *J. Adhesion* **2**, 184–201
14. Sarin, V. K., Kent, S. B. H., Tam, J. P., and Merrifield, R. B. (1981) *Anal. Biochem.* **117**, 147–157
15. Machleidt, W., and Wachter, E. (1977) *Methods Enzymol.* **47**, 263–277
16. Hunkapiller, M. W., and Hood, L. E. (1983) *Methods Enzymol.* **91**, 486–494
17. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 4350–4354
18. Renart, J., Reiser, J., and Stark, G. R. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 3116–3121
19. Gershoni, J. M., and Palade, G. E. (1982) *Anal. Biochem.* **124**, 396–405
20. Horn, M., and Bonner, A. G. (1982) in *Methods in Protein Sequence Analysis* (Elzinga, M., ed) pp. 159–171, Humana Press, Clifton, NJ
21. Rastogi, A. K., Rynd, J. P., and Stassen, W. N. (1976) *31st Annual Technical Conference.* Section 6-B, pp. 1–8, The Society

of the Plastic Industry, Inc., Reinforced Plastics/Composites Institute
22. Pleuddemann, E. P. (1972) *27th Annual Technical Conference.* Section 21-B, pp. 1–7, The Society of the Plastics Industry, Inc., Reinforced Plastics/Composites Institute
23. Bio-Rad Technical Bulletin #1080 (1983), Richmond, CA
24. Pick, U., and Avron, M. (1976) *Biochim. Biophys. Acta* **440,** 189–204
25. Kent, S. B. H. (1982) in *Methods in Protein Sequence Analysis* (Elzinga, M., ed) pp. 205–213, Humana Press, Clifton, NJ
26. Bravo, R. (1984) in *Two-dimensional Gel Electrophoresis of Proteins* (Celis, J. E., and Bravo, R., eds) p. 17, Academic Press, New York
27. L'Italien, J. J., and Kent, S. B. H. (1984) *J. Chromatogr.* **283,** 149–156
28. Nikodem, V., and Fresco, J. R. (1979) *Anal. Biochem.* **97,** 382–386
29. Detke, S., and Keller, J. M. (1982) *J. Biol. Chem.* **257,** 3905–3911
30. Cleveland, D. W., Fischer, S. G., Kirschner, M. W., and Laemmli, U. K. (1977) *J. Biol. Chem.* **252,** 1102–1106
31. Laursen, R. A. (1971) *Eur. J. Biochem.* **20,** 89–102